

Brain anatomy of the 4-day-old European rabbit

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Abstract

The European rabbit (*Oryctolagus cuniculus*) is a widely used model in fundamental, medical and veterinary neurosciences. Besides investigations in adults, rabbit pups are relevant to study perinatal neurodevelopment and early behaviour. To date, the rabbit is also the only species in which a pheromone – the mammary pheromone (MP) – emitted by lactating females and active on neonatal adaptation has been described. The MP is crucial since it contributes directly to nipple localisation and oral seizing in neonates, i.e. to their sucking success. It may also be one of the non-photoc cues arising from the mother, which stimulates synchronisation of the circadian system during pre-visual developmental stages. Finally, the MP promotes neonatal odour associative and appetitive conditioning in a remarkably rapid and efficient way. For these different reasons, the rabbit offers a currently unique opportunity to determine pheromonal-induced brain processing supporting adaptation early in life. Therefore, it is of interest to create a reference work of the newborn rabbit pup brain, which may constitute a tool for future multi-disciplinary and multi-approach research in this model, and allow comparisons related to the neuroethological basis of social and feeding behaviour among newborns of various species. Here, in line with existing experimental studies, and based on original observations, we propose a functional anatomical description of brain sections in 4-day-old rabbits with a particular focus on seven brain regions which appear important for neonatal perception of sensory signals emitted by the mother, circadian adaptation to the short and single daily nursing of the mother in the nest, and expression of specific motor actions involved in nipple localisation and milk intake. These brain regions involve olfactory circuits, limbic-related areas important in reward, motivation, learning and memory formation, homeostatic areas engaged in food anticipation, and regions implicated in circadian rhythm and arousal, as well as in motricity.

Key words: brain anatomy; European rabbit; limbic regions; memory; newborn; olfactory system; social and feeding behaviour.

Introduction

The European rabbit (*Oryctolagus cuniculus*) is a widely used model in neuroscience (fundamental, medical and veterinary) as it has a white/grey matter ratio similar to that of the human brain and exhibits human-like timing of perinatal brain white matter maturation (Derrick et al. 2007). Besides studies in adults, rabbit pups also constitute a well-used model for perinatal neurodevelopment and development of behaviour, ranging from fundamental research,

for instance in brain processing of single odorants and odorant mixtures (e.g. Hudson & Distel, 1986; Allingham et al. 1999; Charra et al. 2012; Schneider et al. 2016a), food anticipatory brain activity, circadian rhythm and sibling interactions (e.g. Caba & González-Mariscal, 2009; Montúfar-Chaveznavia et al. 2012; González-Mariscal et al. 2016; Olivo et al. 2017) and expression of calcium-binding proteins (Wójcik et al. 2013), to medical research, for instance in intrauterine ischaemia (e.g. Derrick et al. 2007), intrauterine growth restriction (e.g. Simões et al. 2015), paediatric traumatic brain injuries (e.g. Zhang et al. 2015), prenatal cocaine exposure (e.g. Gingras et al. 1993) and influence of maternal endotoxin exposure on brain development (e.g. Balakrishnan et al. 2013).

Furthermore, the rabbit is to date the only species in which a pheromone emitted by lactating females and active on neonatal behaviour has been described. Thus, the mammary pheromone (MP; 2-methylbut-2-enal) carried in the female's milk, awakes rabbit neonates and triggers the

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typical orocephalic searching-grasping movements that they usually display during the single daily nursing to locate the nipples and suck (e.g. Coureaud, 2001; Coureaud et al. 2003, 2010; Schaal et al. 2003). As the MP fulfilled the stringent definition of a mammalian pheromone proposed by Beauchamp et al. in 1996 (Coureaud, 2001; Schaal et al. 2003; Coureaud et al. 2010), it offers a remarkable opportunity to determine pheromonal induced-brain processing supporting adaptive behaviour in a newborn mammal. In rabbit pups, such an exploration has been conducted on postnatal days 0 (day of birth; Schneider et al. 2016b) and 4 (Charra et al. 2012), i.e. at an optimal age of pup orocephalic responsiveness to the MP, before progressive decrease and extinction between postnatal days 10–15 and weaning (Coureaud et al. 2006a, 2008a). In addition, while food anticipatory behavioural rhythmicity develops in rabbit pups over the first 4 days after birth (Hudson & Distel, 1982; Jilge, 1993; Montigny et al. 2006), it has been shown that the MP may be one of the non-photoc cues arising from the mother, stimulating synchronisation of the circadian system during pre-visual developmental stages (eyes open between days 10 and 13) (Caba & González-Mariscal, 2009; Montúfar-Chaveznava et al. 2013; Trejo-Muñoz et al. 2014). Finally, the MP also functions as a promoter of neonatal associative and appetitive odour conditioning: only one and brief exposure to a new odour stimulus (single odorant or mixture of several odorants) in the presence of the MP is sufficient to induce the learning of the new stimulus which becomes, 24 h later, a releaser of the sucking-related orocephalic behaviour in pups as efficient as the MP itself (e.g. Coureaud et al. 2006b, 2008b, 2011, 2014a,b); such odour learning may have direct and positive consequences on the neonatal milk intake (Jouhannau et al. 2016). This reinforcing activity of the MP is functional during the first postnatal week of the rabbit pup's life (Coureaud et al. 2010) and investigations of the brain processing supporting the perception of the learned stimulus have started in 4-day-old rabbit pups, both after acquisition of a single odorant presented alone (Charra et al. 2013) and the context of odour mixture perception (Schneider et al. 2016a).

For all these reasons, the rabbit pup is a unique model in neuroscience research, especially regarding chemosensory processes and social and feeding behaviour. However, the literature concerning the rabbit pup brain anatomy is sparse and most of the studies focus on the adult rabbit. Some brain atlases based on histological sections or fMRI images are available for the adult rabbit (histological: Shek et al. 1986; fMRI: Munoz-Moreno et al. 2013; Müllhaupt et al. 2015), but the young rabbit brain remains incompletely described, and studies were often more specialised in mechanisms than in anatomy (e.g. Allingham et al. 1998, 1999; Jagalska-Majewska et al. 2001, 2003a,b; Charra et al. 2012, 2013; Moreno et al. 2013; Schneider et al. 2016a,b). Moreover, fMRI studies only show relatively low resolution images of the brain from 4 to 40 weeks after birth (Lim

et al. 2015), which do not allow study of defined regions important in perception and behaviour. Thus, an anatomical reference document is missing for future multi-disciplinary and multi-approach research using the rabbit pup as a model, as well as for comparative studies (e.g. Sullivan et al. 2000; Rincón-Cortés et al. 2015; Perry et al. 2016). The aim of the present study is to generate a reference document of the functional anatomy of the rabbit pup brain. We propose a general description of histological coronal sections along the rostro-caudal axis of the brain in 4-day-old pups. Our work does not aim to provide an exhaustive overview of the whole brain, but rather focuses on the neuronal substrate underlying the sensory, cognitive and behavioural abilities of rabbit pups at this developmental stage. Therefore, particular attention was paid to the cerebral regions involved in olfactory, limbic, mnemonic processes and feeding behaviour. Data are discussed regarding the existing anatomical knowledge and according to the functional role of the identified brain regions.

Materials and methods

Animals and breeding conditions

New Zealand rabbits (Charles River strain, France) originated from the breeding colony of the Centre de Zootechnie (Université de Bourgogne, Dijon, France). Adult animals were housed in individual cages under a constant 12 : 12 h light : dark cycle (light on at 07:00 am) at 21–22 °C. Food (Lapin Elevage 110, Safe, France) and water were provided *ad libitum*. Two days before expected day of parturition, a nestbox (0.39 × 0.25 × 0.32 m) was fixed to the cages of pregnant females. The day of birth was designated as postnatal day 0. Females had access once a day to the nest for 15 min at 11:30 am, i.e. respecting the short nursing episode displayed by rabbit females both in the wild and in domestic conditions (Zarrow et al. 1965; Lincoln, 1974; Coureaud et al. 2000; González-Mariscal et al. 2016). For the present study, conforming to other anatomical studies and in compliance with ethical constraints, a small number of animals have been used, i.e. four 4-day-old pups (average weight 73.0 ± 7.0 g). They came from four different litters to ensure natural variability between individuals. The observed brain structures were consistent with those considered in our previous studies of the rabbit pup brain (Charra et al. 2012, 2013; Schneider et al. 2016a,b). The study was carried out in accordance with ethical rules enforced by French law and was supported by ethical committee authorisation no. 1608 from the University of Burgundy and no. 01273.01 from the French Ministry of Higher Education and Research.

Tissue collection and staining

Pups were sacrificed by an overdose of pentobarbital (100 mg kg⁻¹) and perfused transcardially with a 0.9% saline solution containing 5% heparin followed by 450 mL of fixative [4% paraformaldehyde and 0.2% picric acid dissolved in 0.1 M phosphate buffer (PB) at pH 7.4] kept at 4 °C. After perfusion, the brain was removed and placed in the fixative for 2 h, after which it was transferred to a postfixative (2% paraformaldehyde and 0.2% picric acid dissolved in 0.1 M PB at pH 7.4) for 24 h at 4 °C. For cryoprotection, the tissue

was embedded in gelatin blocks and immersed in 30% sucrose solution in 0.1 M PB for 48 h. The embedded brains were cut in two parts (Fig. 1, thick line), one containing the olfactory bulb and parts of the frontal cortex and the other containing the rest of the brain, as a block with the whole brain was otherwise too big to be cut with the cryostat. All brain tissue was cut in 50- μ m-thick coronal serial sections using a cryostat (Leica CM3050).

Brain sections were mounted on gelatin-coated slides, stained using crystal violet (0.5% crystal violet and 0.01% glacial acetic acid in aqueous solution), dehydrated and coverslipped with Depex mounting medium for light microscopy. One pup was excluded from later analysis due to insufficient quality of perfusion and resulting damage to brain sections.

Analysis of brain sections

Overlapping photos of the brain sections (left hemisphere) were taken using a Leica Axiophot 2 light microscope and the MERCATOR® software (Explora Nova, France). All the photos obtained of one brain section (range: 45–130 photos/section) were merged to a single one using Microsoft ICE and were cropped using Adobe PHOTOSHOP 7.0.1. Brain section maps were drawn using Adobe ILLUSTRATOR CS. Each square of the brain map scale grid represents 0.5×0.5 mm. The distances between sections are between 0.5 and 2 mm (Fig. 1). Scales do not take into account brain shrinkage due to tissue processing (Dziewiatkowski et al. 2002). Brain regions were mainly identified by comparison with the brain atlases of adult rabbit brain (Girgis & Shih-Chang, 1981; Shek et al. 1986) and other existing literature in pups (Jagalska-Majewska et al. 2001, 2003a,b) or adult rabbits (Varga et al. 2003; Równiak et al. 2007).

Comparison with other species such as mice (Franklin & Paxinos, 2007; Paxinos et al. 2007) and rat (Paxinos & Watson, 1986) was sometimes required to delineate some brain regions.

Anatomical description of the rabbit pup brain and functional considerations

The external day 4 rabbit brain is very similar in shape to the adult rabbit brain (Shek et al. 1986) but appears more cramped in the rostral-caudal axis. This is especially noticeable for the olfactory bulb and the cerebral hemispheres. The rhinal and sagittal fissures are well defined. The olfactory tract is well developed, consistent with its importance at this age. The optic nerve and chiasma are also easily distinguishable in preparation for the opening of the eyes between days 10 and 13 (Gottlieb, 1971; Ripisardi et al. 1975; Coureaud et al. 2008c). The cerebellar vermis and hemispheres and the flocculus are visible.

In the following, we describe and discuss seven brain centres and circuits: (i) main and (ii) accessory olfactory circuits, (iii) limbic-related areas mainly involved in reward/motivation or (iv) in learning and memory, (v) homeostatic-related areas, (vi) areas involved in circadian rhythm and arousal, and (vii) areas engaged in motricity. These seven brain regions were chosen due to the importance of the rabbit pup as a model in olfaction and in other research specialisations.

The olfactory circuits

Rabbits' ears and eyes are closed at birth and remain in this state for several days (Coureaud et al. 2008c). However, the perception

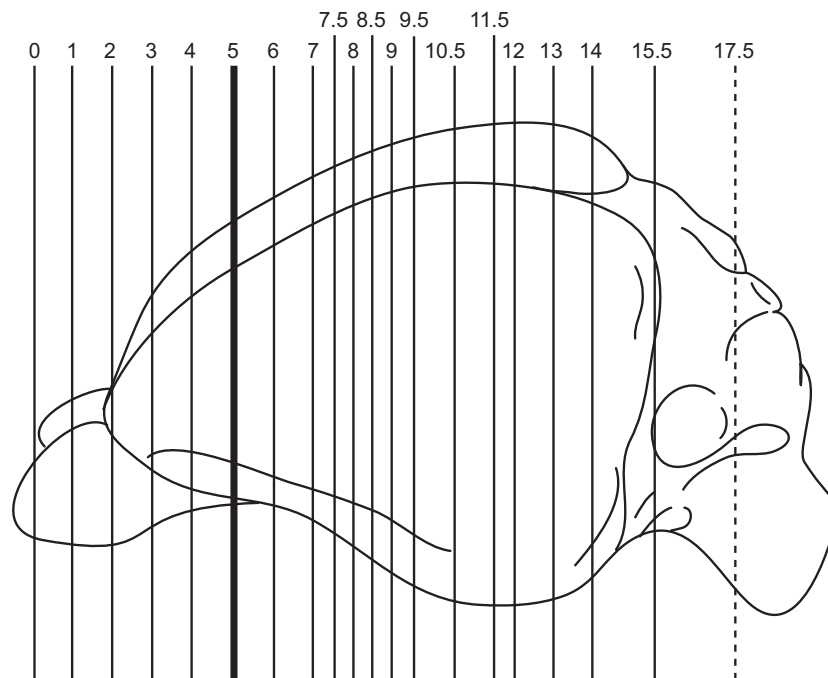


Fig. 1 Schematic lateral view of the brain of a 4-day-old rabbit pup (bodyweight 91.7 g) illustrating the coronal rostro-caudal sections depicted in Figs 2–7. Each brain section was 50 μ m thick. Numbers indicate the distance (in mm) of each of the sections to the most anterior section. The thick line at 5 mm marks where the brain had been cut in two to make brain sectioning possible (the frozen block would otherwise have been too big). The most caudal section was taken from another rabbit pup (bodyweight: 72 g) as this part of the brain was too damaged in the first animal (marked in dotted line). The distance between this most posterior section and the other sections is therefore only approximate and was defined by comparison with sections of the otherwise used rabbit pup.

of odour cues and odour signals, such as the MP, plays a crucial role in the survival of newborn rabbits as they are highly relying on this sense to detect the entry of the mother into the nest and rapidly find the nipples to suck. The olfactory circuits can be separated in the main and the accessory olfactory parts (Scalia, 1966; Broadwell, 1975a; Ojima et al. 1984).

The main olfactory circuit

Olfactory cues are detected by the main olfactory epithelium situated in the nasal cavity which receptor neurons project to the *main olfactory bulb* (MOB) (Table 1, Figs 2.1 to 2.3), which takes up the biggest part of the olfactory bulb. Its coronal sections, rostral to the accessory olfactory bulb, are circular in rabbit pups, whereas in adults they are shaped more like an upside-down pear (Shek et al. 1986). As in adults, the pups' MOB shows six layers, named from the outside to the inside (Broadwell, 1975b; Shek et al. 1986): olfactory nerve layer (ON), glomerular layer (GL), external plexiform layer (EPI), mitral cell layer (Mi), internal plexiform layer (IPI) and granule cell layer (GrO). The characteristic internal structure of the glomeruli (mesh-like arrangement of terminal olfactory neurons) observed in adults, is already present in 4-day-old pups as shown by 3D reconstructions (Katoh et al. 1994). The use of Fos detection as a marker of cellular activation in response to the MP (at day 4: Charra et al. 2012; at day of birth: Schneider et al. 2016b) as to novel or conditioned odours (Allingham et al. 1999; Charra et al. 2013) pinpoints the functionality of the MOB in the first postnatal days. It also seems to contribute to behavioural responsiveness of E27-30 fetuses to the MP (Coureaud, 2001; Schaal et al. 2003) and E30 fetuses to airborne odorants (Hudson, 1985). Furthermore, the MOB

Table 1 Abbreviations of brain regions.

ac	Anterior commissure
aca	Anterior commissure, anterior part
Acb	Accumbens nucleus
AcbSh	Accumbens nucleus, shell
ACo	Anterior cortical amygdaloid area
AD	Anterodorsal thalamic nucleus
AH	Anterior hypothalamic area
AM	Anteromedial thalamic nucleus
AOD	Anterior olfactory nucleus, dorsal part
AOB	Accessory olfactory bulb
AOL	Anterior olfactory nucleus, lateral part
AON	Anterior olfactory nucleus
AOP	Anterior olfactory nucleus posterior
APir	Amygdalopiriform area
Aq	Aqueduct
Arc	Arcuate nucleus
ATg	Anterior tegmental nucleus
AV	Anteroventral thalamic nucleus
BLA	Basolateral amygdaloid nucleus
BMA	Basomedial amygdaloid nucleus
C	Caudate
cb	Cerebellum
cc	Corpus callosum
CA1	CA1 cell layer of the hippocampus
CA2	CA2 cell layer of the hippocampus
CA3	CA3 cell layer of the hippocampus
CE	Central amygdaloid nucleus

(continued)

Table 1. (continued)

Cg	Cingulate cortex
Cl	Clastrum
CN	Cochlear nucleus
CM	Central medial thalamic nucleus
Cp	Cerebral penducle
CPu	Caudate putamen
CxA	Cortex-amygdala transition zone
DG	Detate gyrus
DK	Nucleus of Darkschewitsch
DM	Dorsomedial hypothalamic nucleus
DR	Dorsal raphe nucleus
DS	Dorsal subiculum
DTT	Dorsal tenia tecta
D3V	Dorsal third ventricle
ec	External capsula
En	Endopiriform nucleus
Ent	Entorhinal cortex
EP	Entopeduncular nucleus
EPI	External plexiform layer of the olfactory bulb
EPIA	External plexiform layer of the accessory olfactory bulb
EW	Edinger-Westphal nucleus
f	Fornix
F	Fimbria-fornix
fmi	Forceps minor of the corpus callosum
Fr	Frontal cortex
fr	Fasciculus retroflexus
G	Geniculate nucleus
GIA	Glomerular layer of the accessory olfactory bulb
GI	Glomerular layer of the olfactory bulb
GrA	Granule cell layer of the accessory olfactory bulb
GrO	Granule cell layer of the olfactory bulb
GP	Globus pallidus
Hipp	Hippocampus
ICJM	Island of Calleja, major island
ICx	Insular cortical area
InG	Intermedial grey layer of superior colliculus
IP	Interpenduncular nucleus
IPI	Internal plexiform layer
IPIA	Internal plexiform layer of the accessory olfactory bulb
LA	Lateral amygdaloid nucleus
LC	Locus coeruleus
Cg	Cingulate cortex
LD	Lateralodorsal thalamic nucleus
LH	Lateral hypothalamus
LHb	Lateral habenula
LL	Lateral lemniscus
LPO	Lateral preoptic area
LSD	Lateral septal nucleus, dorsal
LSI	Lateral septal nucleus, intermedial
LSV	Lateral septal nucleus, ventral
LT	Lateral thalamic nucleus
LOT	Lateral olfactory tract
LV	Lateral ventricle
MOB	Main olfactory bulb
Me5	Mesencephalic trigeminal nucleus
MeA	Medial amygdaloid nucleus
MHB	Medial habenula

(continued)

Table 1. (continued)

MiA	Mitral cell layer of the accessory olfactory bulb
Mi	Mitral cell layer of the olfactory bulb
Mn	Mammillary nucleus
MnR	Median raphe nucleus
MnPO	Median preoptic nucleus
MPO	Medial preoptic nucleus
mRt	Mesencephalic reticular formation
MS	Medial septal nucleus
mt	Mammillothalamic tract
NLOT	Nucleus of the lateral olfactory tract
ON	Olfactory nerve layer
OP	Optic nerve layer of superior colliculus
opt	Optic tract
OV	Olfactory ventricle
OVLT	Organum vasculosum of the lamina terminalis
PAG	Periaqueductal grey
PBG	Parabigeminal nucleus
PCx	Piriform cortex
PH	Posterior hypothalamic nucleus
PLCo	Posterolateral cortical amygdaloid area
PLH	Posterior lateral hypothalamus
PL-Pul	Posterior lateral nucleus – pulvinar thalamic complex
PMCo	Posteromedial cortical amygdaloid area
Pn	Pontine nuclei
PPO	Periventricular preoptic nucleus
Pu	Putamen
PVN	Paraventricular nucleus of the hypothalamus
PVT	Paraventricular nucleus of the thalamus
R	Red nucleus
Rbd	Rhabdoid nucleus
RCh	Retrochiasmatic area
RSC	Retrosplenial cortex
Rt	Reticular nucleus
rf	Rhinal fissure
SCh	Suprachiasmatic nucleus
SHi	Septohippocampal nucleus
SN	Substantia nigra
SN5	Spinal trigeminal nucleus
SON	Supraoptic nucleus
sp5	Spinal trigeminal tract
ST	Bed nucleus of the stria terminalis
STh	Subthalamic nucleus
SuG	Superficial grey layer of superior colliculus
Tg	Tegmental nucleus
TT	Tenia tecta
Tu	Olfactory tubercle
3V	Third ventricle
4V	Fourth ventricle
V	Ventro thalamic nucleus
VDB	Nucleus of the vertical limb of the diagonal band
vn	Vomerolateral nerve
VM	Ventromedial thalamic nucleus
VMN	Ventromedial hypothalamic nucleus
VP	Ventral pallidum
VS	Ventral subiculum
VTA	Ventral tegmental area
VTT	Ventral tenia tecta
ZI	Zona incerta

represents a functional circadian pacemaker modulated by the MP and independent of the hypothalamic suprachiasmatic nucleus (SCN) in 7-day-old pups (Montúfar-Chaveznavia et al. 2012, 2013; Trejo-Muñoz et al. 2014). The circadian rhythm may be entrained to mealtimes (Nolasco et al. 2012). The axons of the mitral and tufted neurons from the MOB give rise to the lateral olfactory tract (LOT), which lies ventrolaterally and targets several brain regions which are considered the primary olfactory cortex. Thus, the MOB sends projections to the *anterior olfactory nucleus* (AON) (Figs 2.3 to 2.5) which stretches from inside the caudal end of the olfactory bulb along the whole olfactory peduncle (in pups: this study, in adults: see Young, 1936). The anterior olfactory nucleus is divided into dorsal, lateral and posterior parts (AOD, AOL, AOP respectively), as in adult rabbits. In adults, it has been shown that neurons from this region react to time differences in odour detection between the two nares (Daval & Levetau, 1979). The 1A layer of the *tenia tecta* (TT) (Figs 2.4 and 2.5) is also connected to the MOB in adult rabbits. The TT is located medially to the olfactory ventricle (Broadwell, 1975a) and is divided into a ventral (VTT) and a dorsal part (DTT), which can already be distinguished 4 days after birth. In 4-day-old pups, it has been shown that the TT is differentially activated depending on the odour stimulus (Schneider et al. 2016a). However, the role of this region remains to be investigated in greater detail in the rabbit. Another target area of the MOB is the anterolateral half of the *olfactory tubercle* (Tu; Figs 2.6 to 4.1 here in pups; in adults see Broadwell, 1975a). The Tu is triangular in general outline, lying in the most ventral part of the hemisphere with its apex directed rostrally towards the base of the olfactory peduncle and its base directed caudally to the plane of the optic chiasm in pups (this study) and adults (Young, 1936). The olfactory tubercle is a trilaminar brain area showing a peculiar gyrating structure with anatomically defined 'hills' and 'islands'. Thus, one of the striking features of the olfactory tubercle in rodents and rabbits is the tightly packed cell clusters located dorsally to the dense cell layer, named the islands of Calleja (Meyer et al. 1989). The major island of the islands of Calleja (ICJM) is already distinguishable in the 4-day-old pups (Fig. 3.1). The Tu, which is connected to the reward system, has been shown to be activated during food anticipation in 7-day-old rabbit pups (Olivo et al. 2014). The *nucleus of the lateral olfactory tract* (NLOT) (Fig. 4.2 here in pups), which also receives direct inputs from the MOB, can be distinguished as a rounded nucleus in the most ventral part of the hemisphere at the junction between the anterior commissure and the two hemispheres, between the piriform cortex laterally, and the preoptic area medially (in adults see Young, 1936). In contrast to rodents showing dorsal and ventral parts, the NLOT is rather divided in medial and lateral parts in adult rabbits (Young, 1936; Paxinos & Watson, 1986; Franklin & Paxinos, 2007); these parts, however, can not be distinguished in 4-day-old pups (this study). The MOB also targets the *piriform cortex* (PCx) (Figs 2.4 to 6.2), a paleocortex located laterally and ventrally to the rhinal fissure. This extensive paleocortex is divided in an anterior and a posterior part (aPCx and pPCx) since it starts after the AON rostrally and expands till the lateral entorhinal cortex caudally. The division into aPCx and pPCx in rabbit pups can be made based on the structural changes of the lateral olfactory tract (LOT) (this study), as in mice (Walz et al. 2006). Similar to the adult rabbit (Phillips et al. 1963), the LOT lies lateral to the aPCx, is well developed in its anterior part (Figs 2.6 to 3.1) and becomes less prominent at the level of the anterior commissure in 4-day-old pups (Fig. 3.2). Medially, the aPCx is delimited by the olfactory tubercle, whereas the pPCx is delimited by the cortical amygdaloid nuclei. Both PCx subdivisions exhibit three layers in rabbit pups (I,II,III,

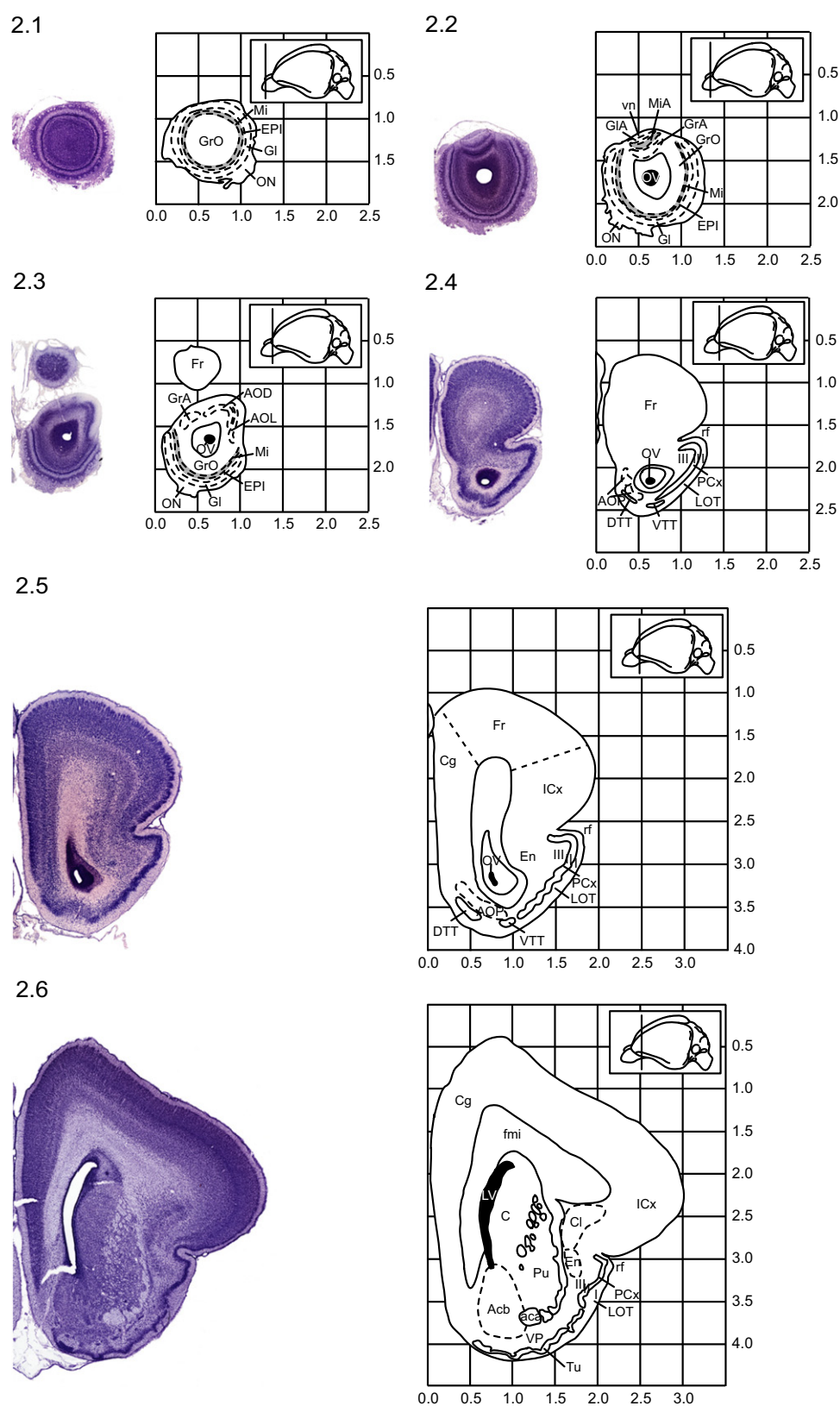
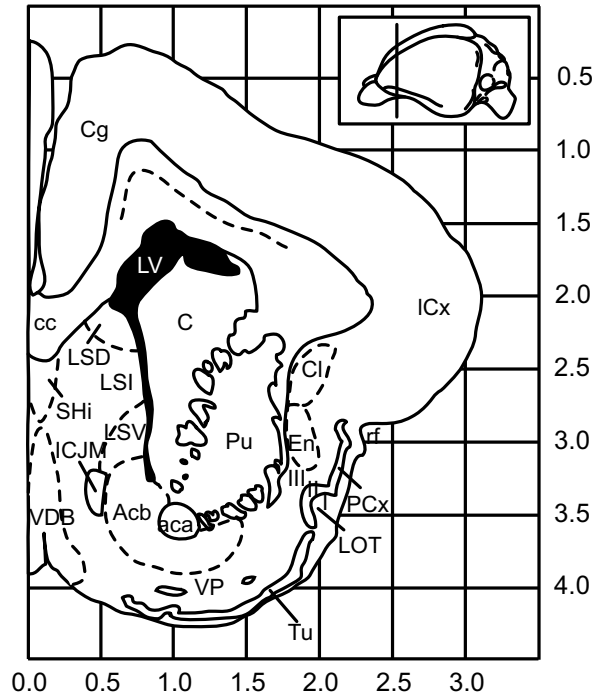


Fig. 2 Brain sections through the 4-day-old rabbit pup's brain stained with crystal violet, from anterior to posterior parts. The sections were chosen to illustrate the main regions of interest used by different research groups currently working on the rabbit pup model. For abbreviations, see Table 1. Each square represents 0.5×0.5 mm.

3.1



3.2

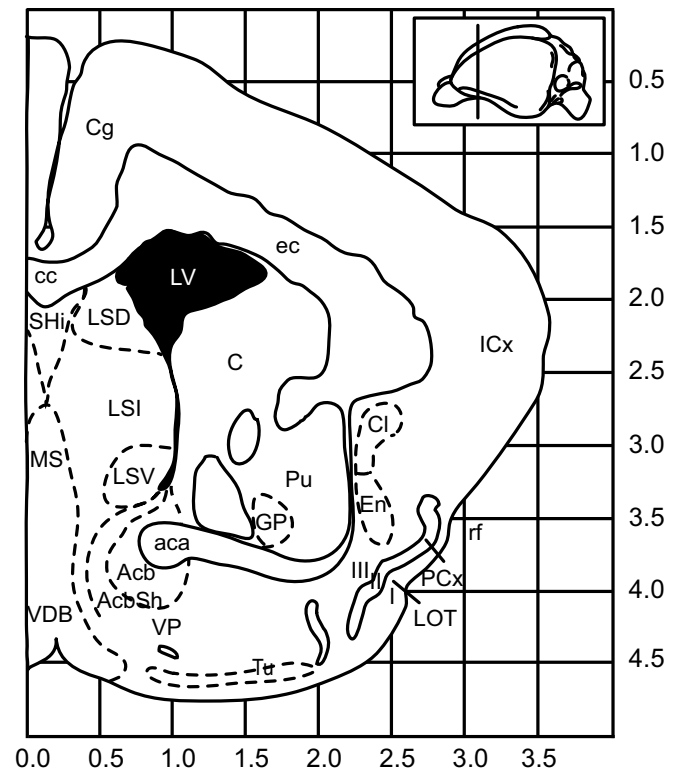
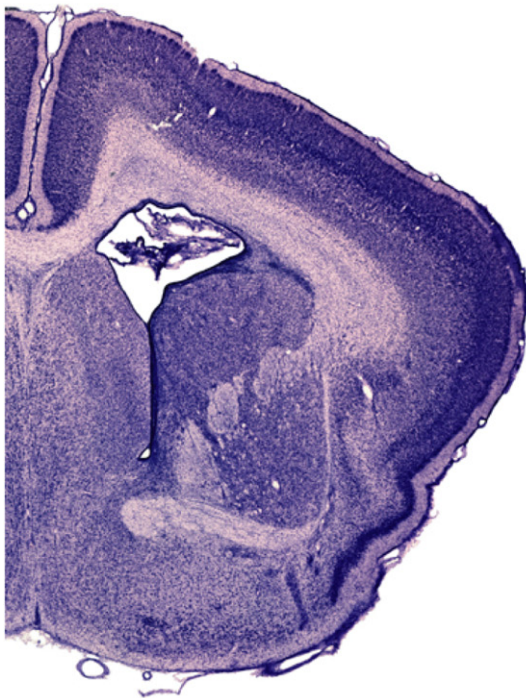
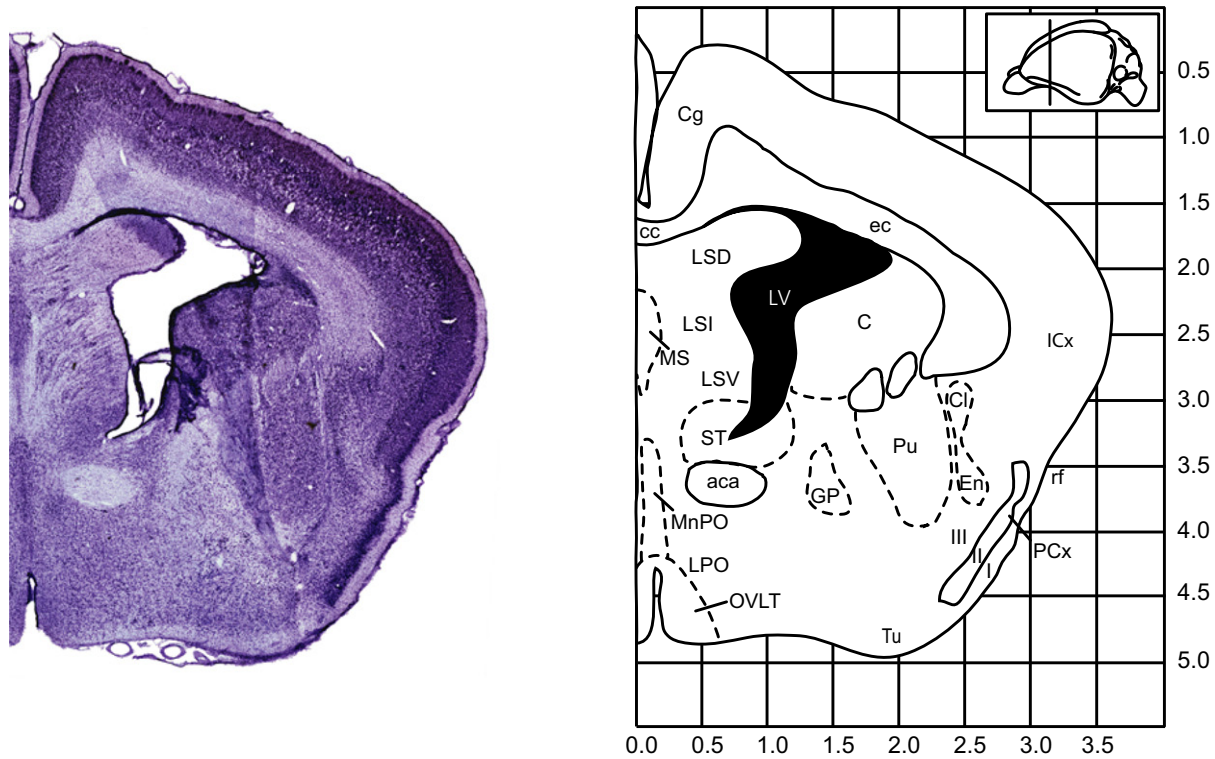


Fig. 3 Brain sections through the 4-day-old rabbit pup brain. See Figure 2 caption.

illustrated in Figs 2.4 to 6.2) as observed in adult rabbits: (i) the superficial plexiform layer, (ii) the superficial compact cell layer and (iii) the deep cell layer (Krakowska et al. 2013). The PCx appears to be involved in perception of the MP and single conditioned odorants presented alone or in elemental/configural mixtures in 0- and

4-day-old pups (Charra et al. 2012, 2013; Schneider et al. 2016a,b) and shows feeding-entrained anticipatory metabolic activity in 7-day-old pups (Olivo et al. 2014). Caudally, the pPCx meets with the entorhinal cortex (Ent) (Figs 7.1 to 8.2). The Ent is a six-layered cortex but layers IV–VI are not easily distinguished here in rabbit pups.

4.1



4.2

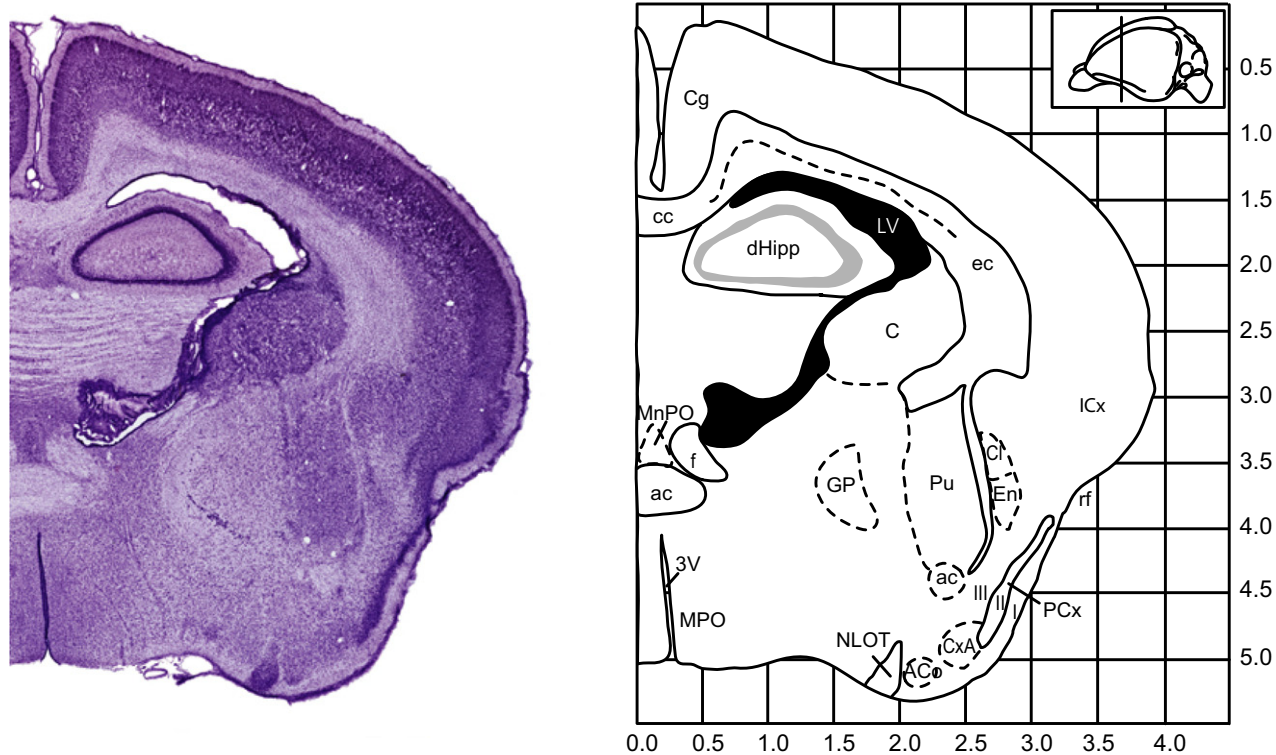
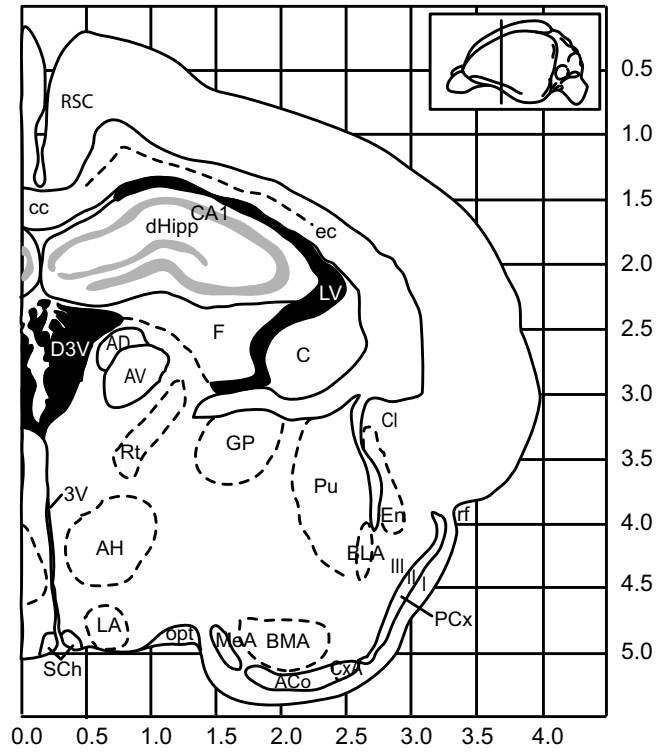
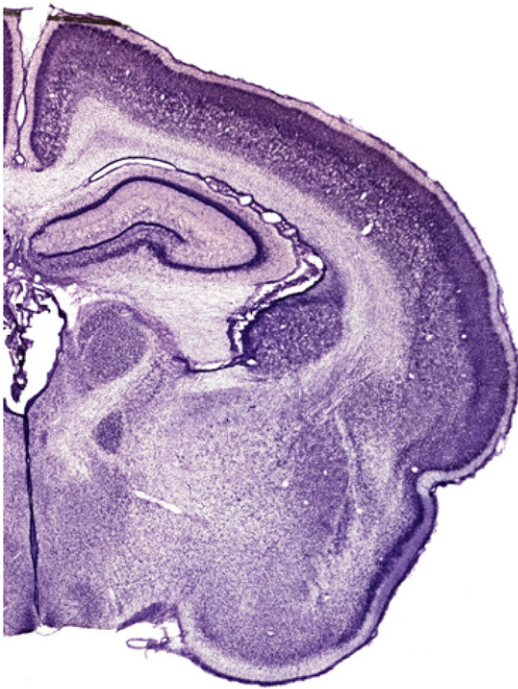


Fig. 4 Brain sections through the 4-day-old rabbit pup brain. See Figure 2 caption.

5.1



5.2

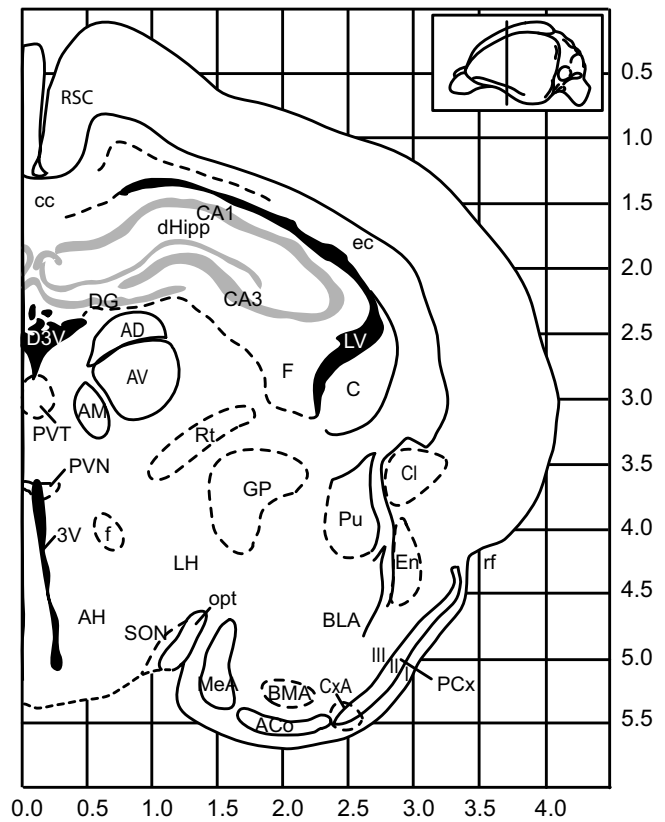
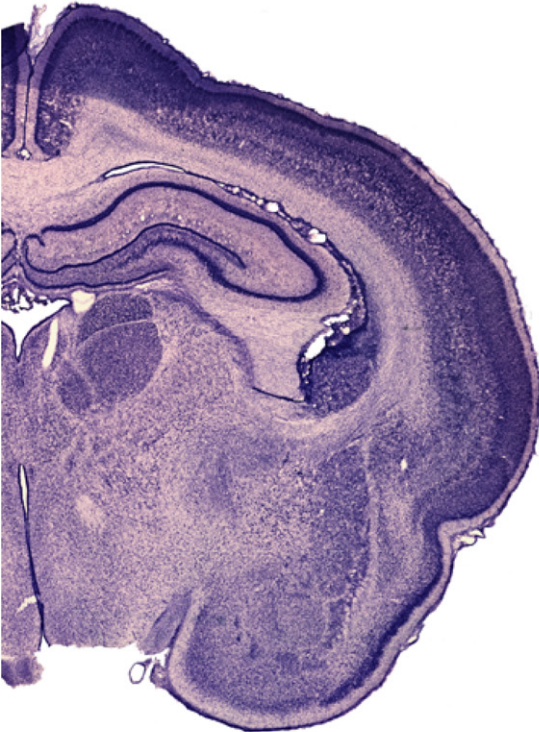
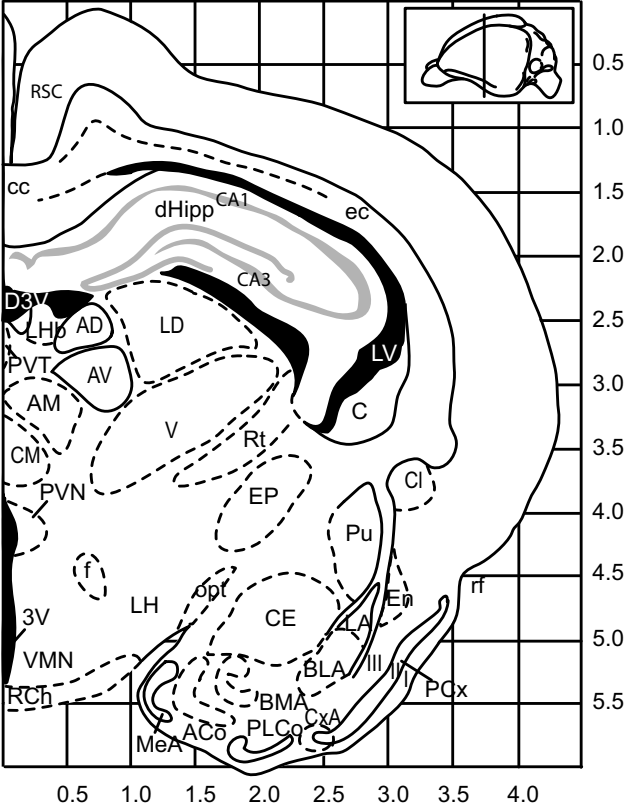
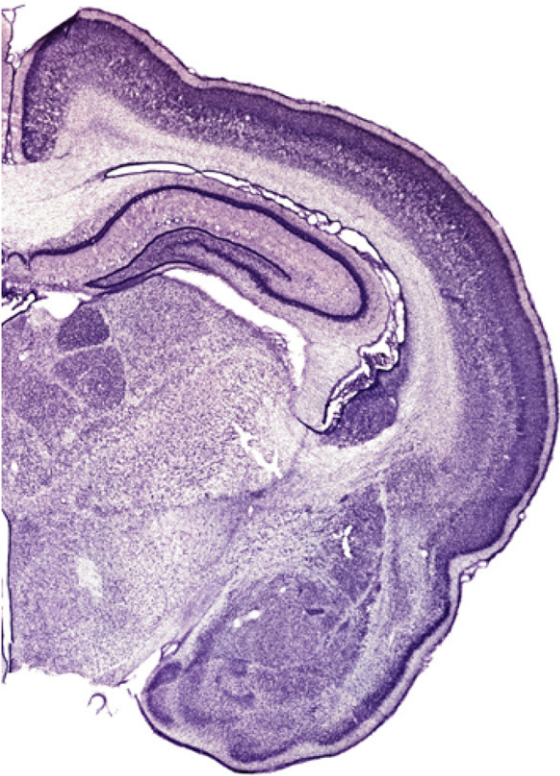


Fig. 5 Brain sections through the 4-day-old rabbit pup brain. See Figure 2 caption.

6.1



6.2

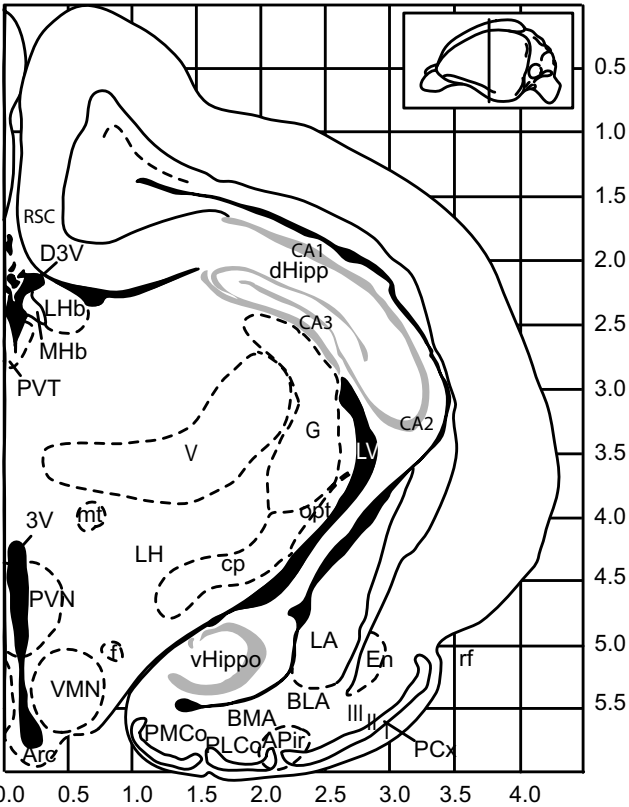
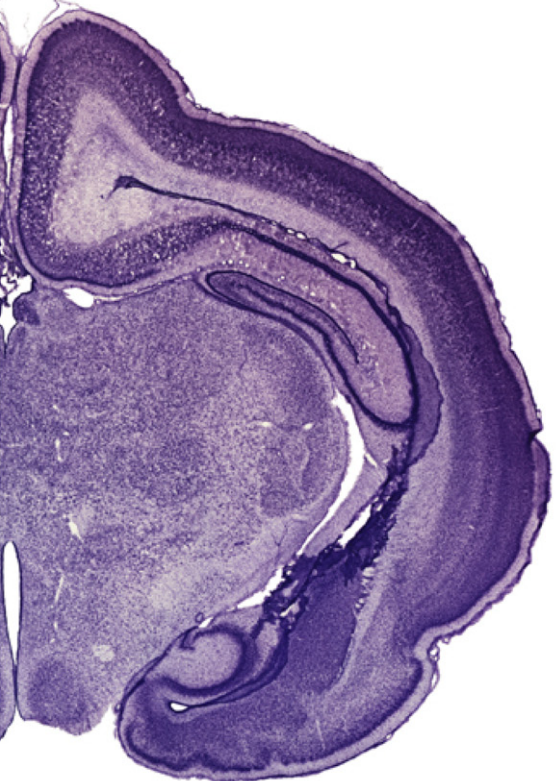
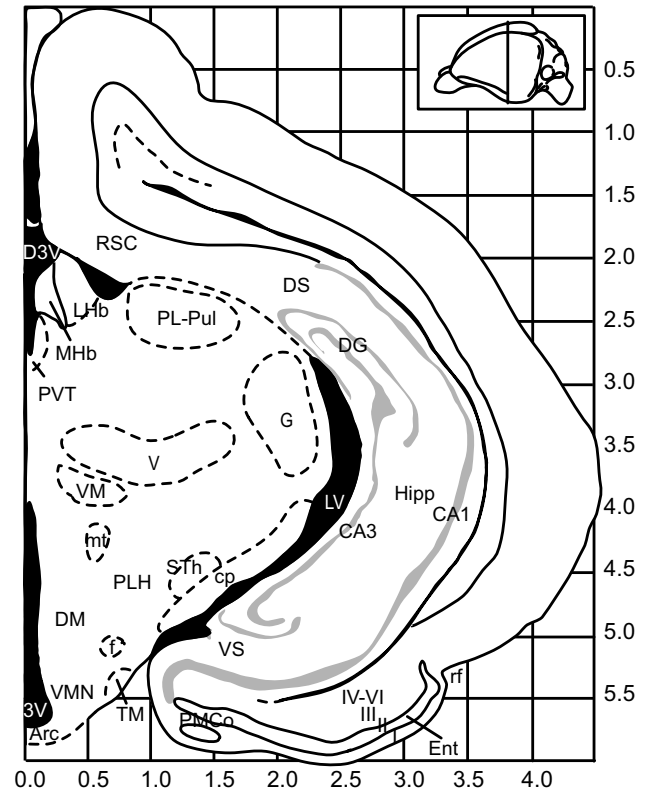
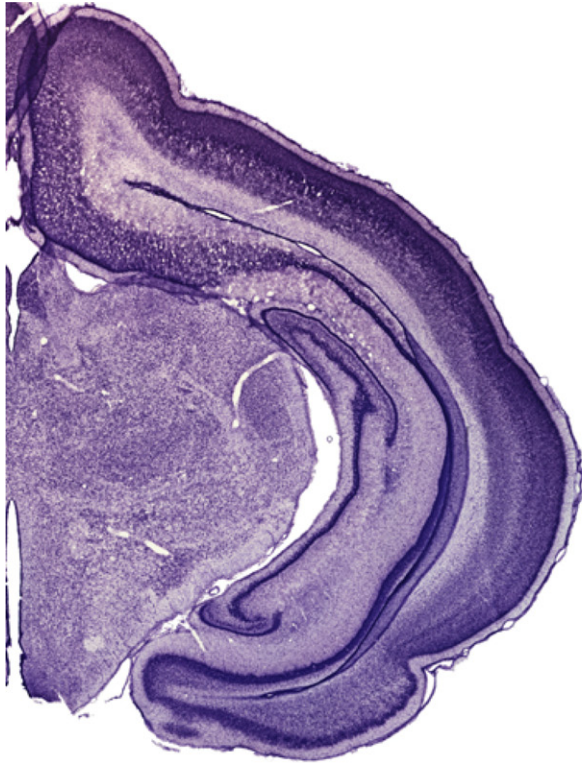


Fig. 6 Brain sections through the 4-day-old rabbit pup brain. See Figure 2 caption.

7.1



7.2

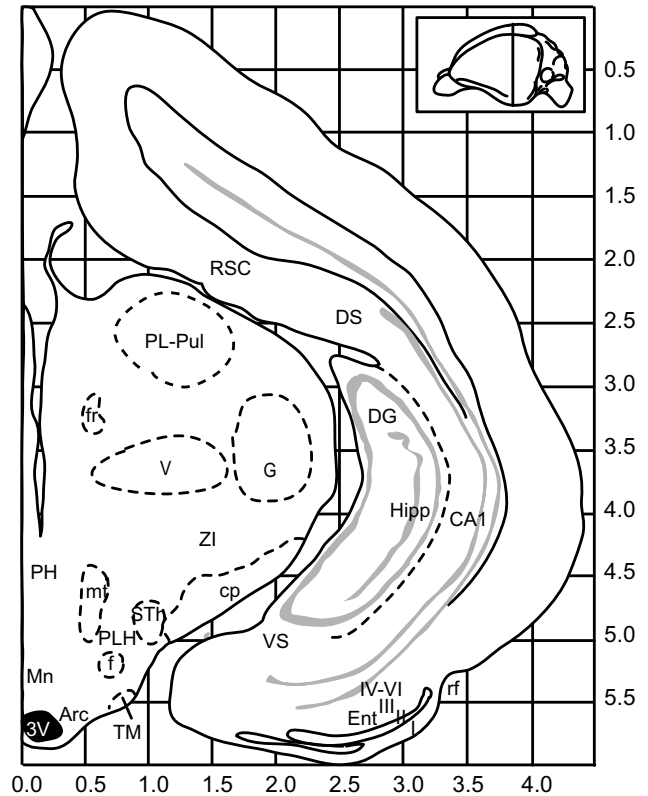
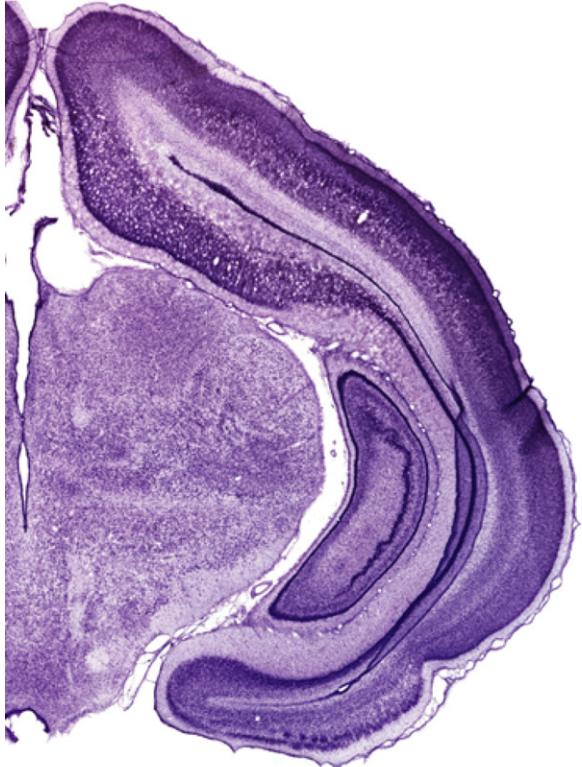
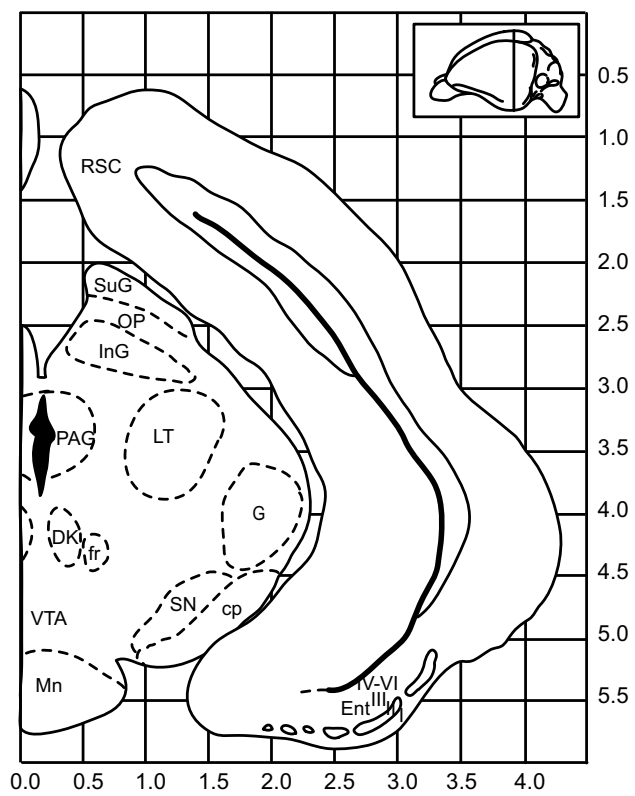
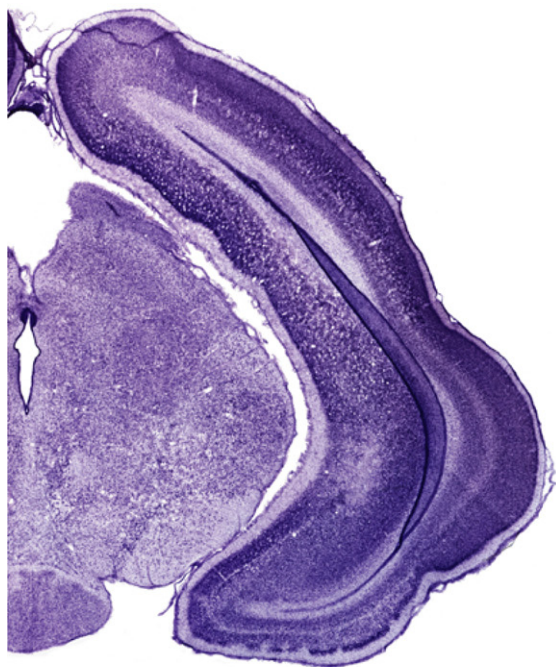


Fig. 7 Brain sections through the 4-day-old rabbit pup brain. See Figure 2 caption.

8.1



8.2

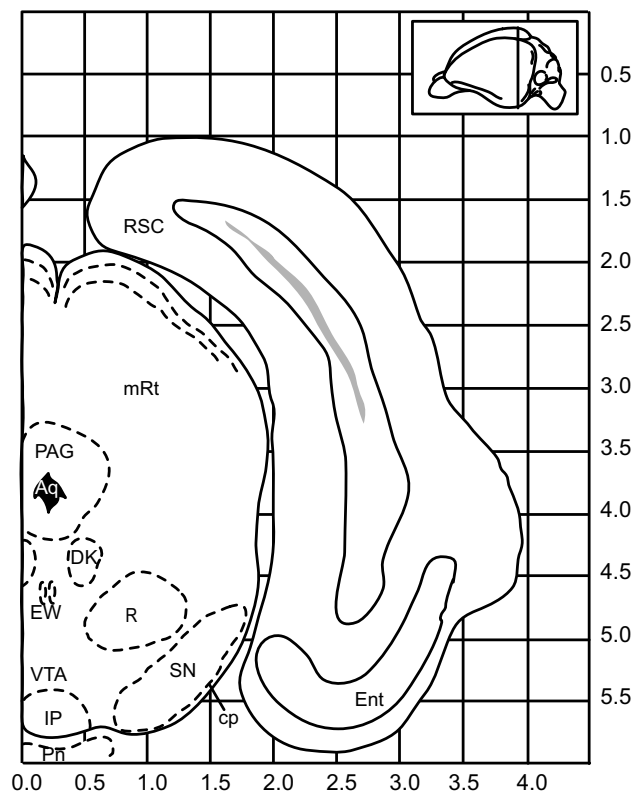
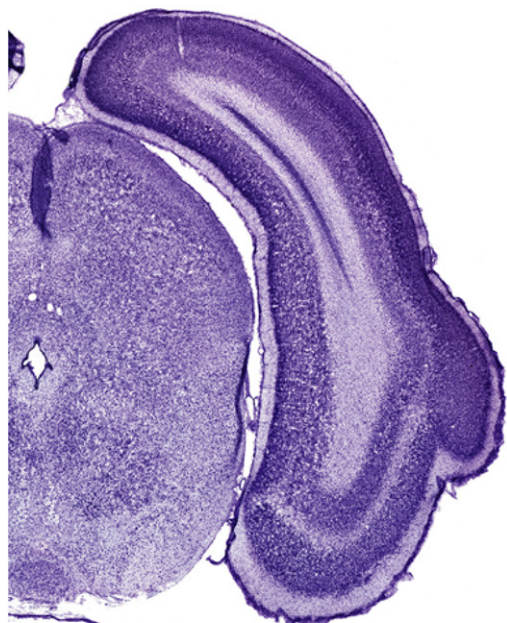


Fig. 8 Brain sections through the 4-day-old rabbit pup brain. See Figure 2 caption.

Moreover, the differentiation into medial and lateral parts cannot be clearly ascertained (Bjarkam et al. 2005). The entorhinal cortex is commonly perceived as a major input and output structure of the hippocampal formation. In rodents as in rabbits, it is the origin of the lateral perforant path that terminates in the hippocampal dentate gyrus (Sancho-Bielsa et al. 2012). Lastly, the amygdala, which is located deep in the temporal lobe, is also highly connected with the MOB. The amygdala is an almond-shaped area which consists of a superficial part and deep nuclei. The superficial nuclei such as the anterior cortical nucleus (ACo) (here in Figs 5.1 to 6.1) and the posterolateral cortical nucleus (PLCo) (Figs 6.1 to 6.2) are the targets of bulbar inputs and play a role in olfactory processing (Scalia & Winans, 1975). The *cortex-amygdala transition zone* (CxA) (Figs 4.2 to 6.1) and the *amygdalopiriform area* (APir) (Fig. 6.2) are also observed in the superficial part of the amygdala. With regard to the various roles of the amygdala in many critical functions, the other nuclei such as the deep nuclei are described in the section 'Limbic-related areas'.

The accessory olfactory circuit

The receptor cells of the vomeronasal organ stretching along to the septum at the bottom of the nasal cavity project to the *accessory olfactory bulb* (AOB) (Figs 2.2 and 2.3), the first central relay of the accessory olfactory circuit, which is embedded dorsally at the posterior end of the MOB, where the olfactory ventricle becomes visible. Its cross-section is ovoid in shape as observed in the adult (Segovia et al. 2006). As for the MOB and as in adults, six layers can be distinguished in rabbit neonates (this study) from the outside: the vomeronasal nerve layer (vn), the glomerular layer (GLA), the external plexiform layer (EPIA), the mitral cell layer (MiA), the internal plexiform layer (IPIA) and the granular cell layer (GrA). Experimentally VNO-deprived rabbit pups can still locate the maternal nipples (Hudson & Distel, 1986). On the day of birth and in 4-day-old pups, the crucial MP signal activates not the accessory pathway but the main olfactory system (Charra et al. 2012; Schneider et al. 2016b). Efferents from AOB mitral cells project to the medial amygdala, which has been shown to transmit olfactory information implicated in social and reproductive behaviour to the hypothalamus in rodents (Asaba et al. 2014). The posteromedial cortical amygdala nucleus (PMCo) (Figs 6.2 to 7.1) also receives direct projections from the AOB and may play a role in odour-guided aspects of reproductive behaviour (Scalia & Winans, 1975; Segovia et al. 2006). The *bed nucleus of the stria terminalis* (ST), sometimes referred to as the extended amygdala, is also a target of the AOB. Anatomically, the ST (Fig. 4.1) is found within the components of the stria terminalis at the level of the anterior commissure. In adult rabbits, the ST can be subdivided into lateral and medial parts (Schwaber et al. 1982) but these subdivisions are not clearly delineated here in 4-day-old pups. The ST projects to the hypothalamus in rodents, allowing monitoring of feeding, drinking and fluid maintenance via brainstem connections (Dong & Swanson, 2006). In adult rabbit females, the ST contains a substantial number of oestrogen receptor-alphas (Caba et al. 2003a) and may be involved in sexual behaviour. In 7-day-old pups, the ST shows a peak of metabolic activity before nursing and is assumed to participate in food anticipatory behaviour (Olivo et al. 2017).

Limbic-related areas

Feeding is crucial for newborn survival, especially in rabbit pups which have only one very brief opportunity (< 5 min) to suck each day, both in domestic and wild conditions (Zarrow et al. 1965;

Hudson & Distel, 1982; Coureaud et al. 2000, 2008c). It is therefore to be expected that regions involved in reward and motivation as well as in learning and memory would be well developed early on. In particular, this concerns odour learning, which occurs perinatally in the rabbit (e.g. Hudson, 1985; Kindermann et al. 1991; Altbäcker et al. 1995; Semke et al. 1995; Allingham et al. 1999; Coureaud et al. 2002; Charra et al. 2013; Schneider et al. 2016a).

Regions mainly involved in reward/motivation

The reward system includes brain areas responsible for incentive salience (i.e. 'wanting', or desire), pleasure (i.e. 'liking', or hedonic value) and positive reinforcement (i.e. learning) (Berridge et al. 2009). In this context, it has been shown to be responsible for triggering appetitive behaviour in various species (Berridge et al. 2009). This system involves several regions as described hereafter.

The *ventral pallidum* (VP) lies just above the olfactory tubercle in 4-day-old pups (Figs 2.6 to 3.2). The separation between the two regions is, however, not clear. The VP seems involved in classical conditioning of the adult rabbit nictitating membrane response (Richardson & Thompson, 1985) but its role in rabbit pups is not established. Together with the VP, the *accumbens nucleus* (Acb) is considered a component of the ventral striatum and appears to play an important role in reward in rodents (Peciña & Berridge, 2005). The Acb (here in Figs 2.6 to 3.2) is located ventral to the lateral ventricle, anterior to the union of both limbs of the anterior commissure, and dorsal to the VP. The Acb can be divided into two distinct areas: a central core surrounded by an outer shell (AcbSh) (Fig. 3.2). In 7-day-old pups, metabolic activity in the Acb peaks during food anticipatory behaviour, whereas after feeding the maximal activity is found in the AcbSh (Olivo et al. 2017). Nearby regions already described in the 'olfactory circuit' such as the Tu, which has a close relationship with the VP, also participate in reward processing. The *lateral septum* found medioventral to the corpus callosum (cc) can be divided into three subregions: the dorsal (LSD), the intermediate (LSI) and the ventral (LSV) lateral septum in pups (here Figs 3.1 to 4.1) as in adults (Meza et al. 2015). It is known to be involved in emotion, fear, aggression, stress, learning and memory in rodents (Jakab & Leranth, 1995). In lactating rabbit does, the lateral septum may be one of the regions sustaining daily nursing rhythm (Meza et al. 2015). Lesions to the septum have strong negative effects on maternal behaviour, although they do not change lactation (Cruz & Beyer, 1972). Similarly, strong metabolic activity prior to feeding during food anticipatory behaviour was observed in 7-day-old rabbits (Olivo et al. 2017).

The *amygdala* is also well-known to be involved in the reward system. The deep amygdala shows two main complexes, basolateral and centromedial (Figs 5.1 to 7.1). In the 4-day-old pup (see also Jagalska-Majewska et al. 2001, 2003a,b at other ages in the young), the amygdala structure resembles that of the adult rabbit (Równiak et al. 2007) but is more compact, with some subregions not identifiable. The basolateral complex consists of the basal and the lateral amygdaloid nuclei. Here, no obvious difference can be seen between the dorsolateral and ventromedial part of the *lateral amygdaloid nucleus* (LA) (Fig. 5.1). At day 4 postpartum, the basal nucleus of the amygdala can be divided into *basolateral* (BLA) (Figs 5.1 to 6.2) and *basomedial nuclei* (BMA) (Figs 5.1 to 6.2), but no further division can be identified, in contrast to 7-day-old pups (Jagalska-Majewska et al. 2003a,b). The centromedial complex is composed of the *central amygdaloid nucleus* (CE) (Fig. 6.1) and the *medial amygdaloid nucleus* (MeA) (Figs 5.1 to 6.1). Wójcik et al. (2013) described differences in the expression of calcium-binding proteins in the developing rabbit amygdala including in 4-day-old

pups. At this early developmental stage, the amygdala is already known to play an important role in food anticipatory behaviour and odour memory. In addition, the BLA and MeA are activated during food anticipatory behaviour in the 7-day-old rabbit pup, although maximal metabolic activity was observed in the cortical amygdala after feeding (Olivo et al. 2017). The BLA receives sensory information from multiple modalities including olfaction, encodes motivationally significant sensory stimuli and is involved in olfactory memory (as observed in rodents; Sevelinges et al. 2009). BLA projections to the lateral hypothalamus (LH) (see section on Homeostatic-related areas) and in rodents to the Acb might sustain reward-related behaviour supporting homeostasis (Ambroggi et al. 2008). Thus, the BLA is critical in the formation of memories of both positive and negative odour cues. In accordance, Charra et al. (2013) reported an activation in 4-day-old rabbit pups of the basal amygdala nucleus following a single associative conditioning to a new odorant paired with the MP.

In the vicinity of the dorsal part of the thalamus, lies the *habenula*. In the rabbit, both in adults (Cragg, 1961) and in pups (this study: Figs 6.1 to 7.1), the habenula can be divided into two parts: a medial one (MHb), which borders the dorsal part of the 3rd ventricle, and a lateral one (LHb). In rodents, the LHb receives olfactory information from the olfactory cortex and has been shown to be involved in the regulation of various motivated behaviours (Sutherland, 1982). In accordance with the role of LHb in motivated behaviour, Charra et al. (2012) showed an increased Fos expression in the LHb in 4-day-old rabbit pups exposed to the MP. The *ventral tegmental area* (VTA) (Fig. 8.1 and 8.2), known for its dopaminergic neurons, is a mesencephalic region located in the ventral midline, dorsal to the interpeduncular nucleus, in a small midline peak extending into the central grey layer between the cell columns of the oculomotor nucleus and among fibres of the third brain nerve (in adults: Kline & Felten, 1985). The neuronal features of the VTA have been described in young (3–21 days) and adult rabbits (Kline & Felten, 1985). Beside its key position in the reward system, the VTA also seems to be involved in conditioned fear response to stressful events in the adult (Guarraci & Kapp, 1999). Laterally to the VTA, the substantia nigra (SN) (Fig. 8.1 and 8.2) is observed, which is also an essential region for dopaminergic neurons.

Regions mainly involved in learning and memory

The memory of chemosensory cues and their association with internal states is highly relevant for the newborn rabbit and its suckling-related adaptive abilities (e.g. Hudson, 1985; Kindermann et al. 1991; Altbäcker et al. 1995; Allingham et al. 1999; Coureaud et al. 2002; Charra et al. 2013; Jouhanneau et al. 2016; Schneider et al. 2016a). The *hippocampus* (Hipp) (Figs 4.2 to 7.2) is a key region for learning and memory processes, as shown in eye-blink conditioning experiments in the adult rabbit (Moyer et al. 1990). As in rodents, the rabbit hippocampus consists of several regions (Berger et al. 1980). The postnatal development of the CA1 region of rabbit hippocampus has been examined by some authors (Schwartzkroin, 1981; Schwartzkroin et al. 1981), indicating a late development of neuronal inhibition processes. In the adult rabbit, Sancho-Bielsa et al. (2012) further examined the organisation of the dentate gyrus (DG) (visible here in 4-day-old pups on Figs 5.2 to 7.2), which is the main entrance gate for information to the hippocampus. According to our study, the gross anatomy of the dorsal (dHipp) and ventral hippocampus (vHipp) in the young resembles that of the adult (Berger et al. 1980), showing differently dark-stained cell layers (Figs 4.2 to 7.2). Although the CA1 and CA3 cell layers can be distinguished, the position of the rather small CA2 cell layer cannot be precisely

established in 4-day-old pups (Fig. 6.2 only shows its approximated position). One may note that, at this age, the vHipp has been shown to be activated by a conditioned odorant perceived in an odour mixture (Schneider et al. 2016a). The *cingulate cortex* (Cg) (Figs 2.4 to 4.2), which receives input from the hippocampus (Vogt et al. 1986) is also a region known to play a role in learning and memory processes (e.g. in eye-blink conditioning in adult rabbits; Oswald et al. 2010). The Cg generally refers to the strip of cortex around the *corpus callosum*. A recent study was able to establish position and delineation of its subdivisions in the adult rabbit (Vogt, 2016). Information concerning the exact positioning of these subdivisions in the 4-day-old rabbit is still missing. The most caudal subdivision of the Cg, the *retrosplenial cortex* (RSC) (Figs 5.1 to 8.2), is a major source of input to parahippocampal areas such as the medial entorhinal cortex. As a result, the RSC is well positioned to have a significant influence on learning and memory. In the adult rabbit, lesions in the RSC have been shown to impair learning of the nictitating membrane response (Berger et al. 1986). The hippocampus projects via the fornix to the *mammillary nucleus* (Mn) (Figs 7.2 and 8.1), which is found on the posterior ventral surface of the brain and can be subdivided in the adult into medial and lateral nuclei (Poremba et al. 1994; however, it was not possible to observe these subdivisions in rabbit pups). Projections from the Mn connect to the *anterior thalamic nuclei* (AD, AM, AV) (Poremba et al. 1994), which are found dorsal of the hypothalamus and are here visible as more densely stained cell clusters adjacent to the third ventricle in 4-day-old pups (Figs 5.1 to 6.1). The AV can be further divided into the parvocellular and the magnocellular divisions in the adult rabbit (Shibata & Yoshiko, 2015). It is important in mediating learning of a discriminative avoidance in adult rabbits (Gabriel, 1990, 1993).

Homeostatic-related areas

The *organum vasculosum of the lamina terminalis* (OVLT) is a circumventricular organ that plays an important role in homeostasis. It is found in the anterior wall of the optic recess of the third ventricle anterior to the hypothalamus (here Fig. 4.1 in 4-day-old pups) and possesses special permeability characteristics important for its involvement in osmoregulation (Yamaguchi et al. 1993; Badoer et al. 1997). Selective activation has been observed in this region after MP exposure in 4-day-old pups (Charra et al. 2012) but not on the day of birth (Schneider et al. 2016b). Food anticipatory activity has been also shown to activate the OVLT at nursing in 7-day-old rabbit pups (Moreno et al. 2013). Beside the OVLT, the hypothalamus is also a key region for homeostatic processes. Indeed, the hypothalamus integrates information from sensory, endocrine, visceral and somatomotor systems in order to control energy balance, thermoregulation, metabolic activity and regulation of the cardiovascular system (Evans, 1976; Gellman et al. 1981; Shoham et al. 1989; Garcia-Garcia, 2012). Briefly, it is involved in drinking, feeding, reproduction, maternal, defensive and aggressive behaviours (Schwartzbaum, 1988; Schwartzbaum & Leventhal, 1990; Girolami et al. 1997; González-Mariscal, 2001; Garcia-Garcia, 2012). The hypothalamus is found in the ventral half of the diencephalon, on either side of the third ventricle (here in Figs 5.1 to 7.2). It starts anterior at the level of the anterior commissure and stretches posterior until the start of the aqueduct in both adults and 4-day-old pups. The hypothalamus has vascular and neuronal connections to the pituitary and neuronal connections with all major parts of the brain and spinal cord (Harris, 1947; Evans, 1976; Blessing et al. 1982). The preoptic area is located in the anterior part of the hypothalamus, surrounding the third ventricle (Bogus-Nowakowska

et al. 2006). In the rabbit, it is important in sexual behaviour, maternal behaviour, sleep regulation and thermoregulation (Burikov & Suntsova, 1989; Shoham et al. 1989; Caba et al. 2003a; Meza et al. 2015). Four regions can be distinguished in the preoptic area of the adult rabbit (Bogus-Nowakowska et al. 2006): the *periventricular preoptic nucleus* (PPO) just adjacent to either sides of the ventricle, followed more laterally by the *medial preoptic nucleus* (MPO) and then by the *lateral preoptic region* (LPO); the fourth part is the *median preoptic nucleus* (MnPO), which is found dorsal to the third ventricle. The present study indicates that the last three regions can also be distinguished in 4-day-old pups (MPO in Fig. 4.2, LPO and MnPO in Fig. 4.1). At this latter age, exposure to the MP induces an activation of the LPO in comparison with exposure to a behaviourally neutral odorant (such activation is absent in pups on the day of birth before any natural exposure to the MP in milk; Charra et al. 2012; Schneider et al. 2016b). Neither the MnPO nor the MPO were specifically activated by the MP (Charra et al. 2012; Schneider et al. 2016b), but nursing induced a significant increase in Fos expression in these regions (Moreno et al. 2013). The MnPO appears to be involved in food anticipatory activity as well: Moreno et al. (2014) observed robust clock protein oscillations in pups close to being nursed and high levels of clock protein during food anticipatory activity.

Furthermore, the hypothalamus contains neurosecretory neurons located in the *supraoptic* (SON) and *paraventricular* (PVN) nuclei. Thus, magnocellular neurons of the SON and PVN synthesise vasopressin and oxytocin (also known as neurohypophysial hormones) secreted from the posterior pituitary into the systemic circulation (Schimchowitsch et al. 1989). Oxytocin plays an essential role in reproduction and mother–young bonding (Aulsebrook & Holland, 1969; Febo et al. 2005). Vasopressin is synthesised in response to signals generated by osmoreceptors and is involved in the regulation of water ingestion and thirst (Sundsten & Sawyer, 1961). Here, in 4-day-old rabbit pups, the PVN (Figs 5.2 to 6.2) forms two triangular, more densely stained areas on either side of the dorsal part of the third ventricle (Soliman et al. 2015). The PVN plays an essential role in the control of food intake and energy expenditure by integrating multiple neural and humoral inputs (Prior et al. 2014). The activity increases in the magnocellular neurons of the PVN during periods of anticipatory arousal in 7-day-old rabbits; moreover, while suckling decreases activity of magnocellular neurons, it increases the activity of parvocellular neurons (these expression patterns become more distinct in pups from 3 to 7 days, suggesting maturational or experience-related influences; Allingham et al. 1998). Furthermore, milk intake or stroking increases activation of oxytocinergic and vasopressinergic neurons in the anterior and medial PVN (Caba et al. 2003b). Finally, the PVN shows feeding entrained rhythmic increase of neuronal activity and expression of the clock protein PER1 (Morgado et al. 2011). Regarding the vagal-hypothalamic axis, it appears to be at least mature in 7-day-old pups (Caba et al. 2003b). Here, the SON (Fig. 5.2) can be distinguished in 4-day-old pups, adjacent to the optic tract on its dorsomedial and lateral side, as in 7-day-old (Allingham et al. 1998) and adult rabbits (Soliman et al. 2015). C-Fos expression has been observed to increase in the SON of 3- and 7-day-old pups (Allingham et al. 1998; Caba et al. 2003b). However, this increase does not appear to be related to food anticipatory arousal alone. The SON shows rhythmic increase of neuronal activation, and expression of a clock protein induced through scheduled feeding (Morgado et al. 2011). The SON only showed increase activation of oxytocinergic neurons after nursing, but not after stroking only, whereas increased activation of vasopressinergic neurons could be observed after both actions (Caba

et al. 2003b). In the rat, neurons from the SON as well as the PVH receive projections arising from the OVLT, allowing activity regulation of vasopressin-secreting neurons (Yang et al. 1994; Grafe et al. 2014).

More caudally, our results allow the *arcuate nucleus* (Arc) to be located in the ventromedial hypothalamus on either side of the third ventricle connected via the median eminence (Figs 6.2 to 7.2). The *ventromedial hypothalamic nucleus* (VMN) is situated just dorsal to the Arc and is more darkly stained (Figs 6.1 to 7.1). The *dorso-medial hypothalamic nucleus* (DM) is found even more dorsally and adjacent to the ventricle (Fig. 7.1). The DM is implicated in timing of food-entrained behaviour and shows food-entrained clock protein expression peaks in 8-day-old pups (Caba et al. 2008). The *anterior hypothalamic area* (AH) appears on either side of the third ventricle anterior of the VMN (Figs 5.1 and 5.2).

According to our results, the *lateral hypothalamus* (LH) is situated lateral to the mammillothalamic tract and the fornix, laterally limited by the optic tract (opt) and the cerebral pendule (cp) (Figs 5.2 to 6.2). This region contains neurons that synthesise the neuropeptides called orexins, known to be essential for arousal and feeding behaviour in mammals (Sakurai, 2014). Orexinergic neurons in the LH are differentially activated between fasted and nursed 7-day-old rabbit pups (Moreno et al. 2013). LH and its anatomically connected areas may play an essential role in the execution of odour-driven motivated behaviours such as consumption of preferred foods/orientation toward natural rewards (Schwartzbaum, 1988). In newborn rabbit, on the day of birth, the LH orexinergic neurons are activated by either a neutral odour or the MP, suggesting a role of these neurons in a change of arousal induced by any odour cue (Schneider et al. 2016b). The study by Schneider et al. pointed out differences between odour treatment groups observed in Fos only-expressing LH neurons but not in Fos/Orexin-expressing neurons, leaving the question of which neurons are specifically involved in MP sensing in the LH to be answered. The role of the LH as a key region in sensing physiological and chemosensory signals for basic survival needs is well-established. Caudally, our present results indicate that the LH extends with the *posterior lateral hypothalamus* (PLH) (Fig. 7.1 and 7.2) and the *posterior hypothalamic nucleus* (PH) (Fig. 7.2).

Circadian rhythm and arousal

The circadian rhythms control a wide variety of physiological events, including metabolism, in all organisms (Eckel-Mahan & Sassone-Corsi, 2013). The *suprachiasmatic nucleus* (Sch), the locus of the master circadian clock, is a small nucleus classically described as situated in the ventral hypothalamus just above the optic chiasm on either side of the third ventricle (Caba et al. 2008; Morgado et al. 2011). The staining method used here did not allow the Sch to be localised in 4-day-old pups. In the rabbit, 7-day-old pups already show a robust circadian rhythm of the clock protein PER1 and clock genes in the Sch (Caldelas et al. 2007; Caba et al. 2008). The rhythmic expression of PER1 and neuronal activation are not greatly influenced by the feeding schedule (Morgado et al. 2011). Projections from the Sch reach other regions strongly involved in the circadian rhythm, such as the *paraventricular thalamic nucleus* (PVT) and the *tuberomammillary nucleus* (TM), as observed in the Nile grass rat (Schwartz et al. 2011). The PVT is known to receive projection from orexinergic neurons of the LH and is a part of the circuitry supporting wakefulness in rodents (de Lecea et al. 1998).

In our 4-day-old rabbits, the PVT was found dorsal of the medial thalamus lining the third ventricle (Figs 5.2 to 7.1). This region

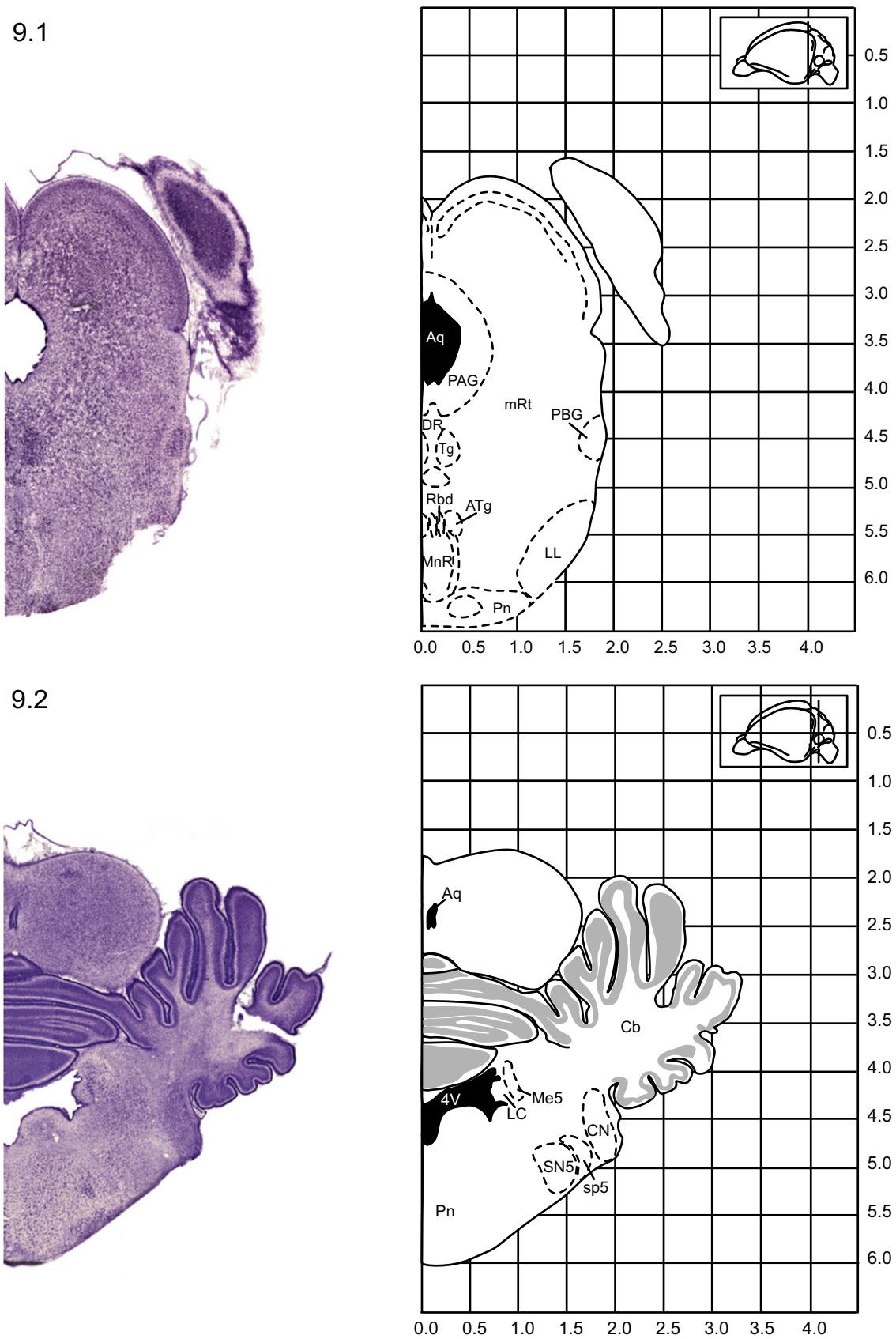


Fig. 9 Brain sections through the 4-day-old rabbit pup brain. See Figure 2 caption.

shows increased neuronal activation 2 h after nursing or expected nursing in 7-day-old pups (Allingham et al. 1998) and is involved in circadian nursing-anticipatory activity (Caba & González-Mariscal, 2009). The TM, as the Mn (see 'Limbic-related areas'), appears to be involved in sleep, arousal, learning and memory as well as feeding and energy balance in rodents (Huston et al. 1997; Valdés et al. 2005; Thakkar, 2011). The TM is located within the posterior third of the hypothalamus ventral to the fornix, as observed here (Fig. 7.1 and 7.2). It consists of a magnocellular cell cluster which is the main source of neuronal histamine in the brain. In this region, even 7-day-old rabbits already show rhythmic neuronal activation and expression of clock protein PER1 induced by a feeding schedule (Morgado et al. 2011).

Further brain regions are involved in arousal, e.g. in preparation of a meal, such as the *raphe nucleus* and the *locus coeruleus* (LC). The different parts of the raphe nucleus in the adult rabbit were described by Felten & Cummings (1979). In rabbit pups, it appears possible here to distinguish the *dorsal raphe nucleus* (DR), which is found on the midline of the brain stem just ventral of the periaqueductal grey (PAG), and the *median raphe nucleus* (MnR), which is situated dorsal to the pontine nuclei (Pn) (Fig. 9.1). The LC is here located at the ventrolateral edge of the fourth ventricle in the young (Fig. 9.2) and in adult rabbits (Felten & Cummings, 1979; Segovia et al. 2006). The LC neurons are less packaged and the borders with the nucleus of the trigeminal nerve are more diffuse than in rodents (Segovia et al. 2006). While isomorphic at morphological level, noradrenergic function of the LC has been reported to be sexually dimorphic (Yang et al. 1996). Two parts, a dorsal and a ventral one, can be distinguished in the adult (Segovia et al. 2006), but so far not in the rabbit pup. The LC is the target of orexinergic fibres from the LH, and participates in sleep–wake regulation in rodents (de Lecea et al. 1998).

Motricity-related areas

Many parts of the brain are involved in the acquisition, representation, execution and control of motricity in mammals. Two main brain areas related to the regulation of movements (previously elaborated in higher cortical regions) are the basal ganglia and the *cerebellum* (Cb; Middleton & Strick, 2000). Together with the motor cortex and the prefrontal cortex, they form discrete motor and cognitive circuits (Middleton & Strick, 2000). The basal ganglia are involved in learned habitual action in mammals (Seeger & Spiering, 2011). Some important parts of the basal ganglia are the *caudate* (C), the *putamen* (Pu), the *globus pallidus* (GP) and the *substantia nigra* (SN) (Middleton & Strick, 2000). Besides being involved in the control of motor movement, C and Pu are also known to play a role in reward and decision-making in mammals (Balleine et al. 2007). In the rabbit, the C and Pu may be already distinguished in 4-day-old pups (Figs 2.6 to 6.1). They can be found in the anterior brain just dorsal of the forceps minor of the *corpus callosum* (fmi), on the lateral side of the lateral ventricle, and are separated by the capsula interna. Both these regions have been shown to contain cholinergic neurons in the rabbit (Varga et al. 2003) and to exhibit maximal metabolic activity after feeding in 7-day-old pups (Olivo et al. 2017). The GP can be observed here as medially adjacent to the Pu (Figs 3.2 to 5.2) as also observed in adult rabbits (Varga et al. 2003). It consists of two major segments, external (or lateral) and internal (or medial) in adults (Wasilewska et al. 2002), which are not observable here in 4-day-old pups. As can be seen in Fig. 8.1 and 8.2, the SN is found in the midbrain. It consists of two parts with very different connections and functions, the *pars compacta* and the *pars*

reticulata; however, these could not be distinguished here in pups, in contrast to adults (Gioli et al. 1985). Dopaminergic neurons projecting from the *pars compacta* of the SN to the C and Pu form the nigro-striatal pathway (Gioli et al. 1985). Lesions of the nigro-striatal dopaminergic pathway retard Pavlovian somatomotor learning without affecting concurrent autonomic learning (Kao & Powell, 1988). In the rabbit, the SN is also involved in the motor regulation of eye movements (Neverov & Bures, 1979).

In the ventral back part of the brain, 4-day-old rabbit pups present a *cerebellum* (Cb) divided into two hemispheres, which also contains a narrow midline zone called the vermis (Fig. 9.2). The cerebellum is usually considered to be involved in controlling movement, balance, learning and proprioception (Brodal, 1940; Manto et al. 2012). In adult rabbits, the Cb is necessary for classical eye-blink conditioning with a non-somatosensory unconditioned stimulus (Rogers et al. 1999). Different lobes can be observed in rabbit pups and adults, and they do look different, especially in the dorsal median part (this study, and see Brodal, 1940 in adults). Its gross and cellular morphology (Lossi et al. 1995) suggests that the Cb is not fully developed here in 4-day-old pups.

Conclusions

Our purpose was to contribute to a better description of the brain anatomy of the rabbit in the first postnatal days. At this period, the developmental state of the rabbit pups' brain is sufficient to support the expression of behaviours essential for survival, i.e. food anticipation, olfactory-related arousal, milk source detection, location and oral seizing, milk intake and thermoregulation. Notably, essential connections/relationships between the circuits of olfaction, food intake and memory appear to be already established at least here at postnatal day 4. The present study, which is the first extensive description of the rabbit pup brain anatomy at this age, could be used in the future to help perform experiments dedicated to the study of brain functions. In particular, the model of the rabbit pup is highly relevant to investigate the development of olfaction, arousal, motivation, reward, learning/memory, motricity, circadian rhythm, and feeding and social behaviour.

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